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## Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	8
References.....	9
Appendices.....	9

## Introduction

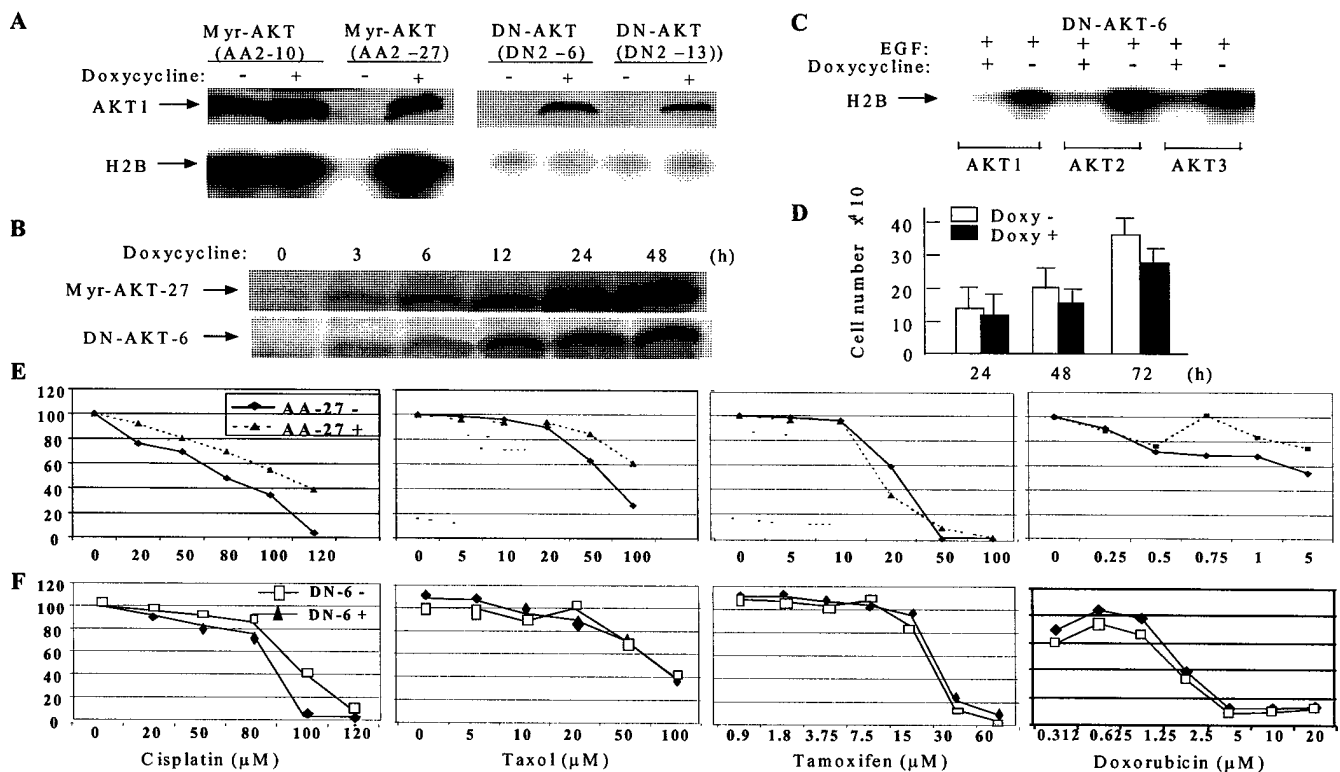
The purpose of this project is to: 1) determine the incidence and clinicopathological significance of *PI3K/AKT2* alterations in breast cancer, 2) examine the role of overexpression of active and wild type *PI3K/AKT2* in mammary cell transformation and 3) determine the role of *PI3K/AKT2* in chemoresistance and as targets for breast cancer intervention.

## Body:

During the last budget year, we have mainly focused our effort on specific aim 3, i.e., determine the role of *PI3K/AKT2* in chemoresistance and as targets for breast cancer intervention.

### 1. *PI3K/AKT2* activation contributes to chemoresistance.

We have previously shown significant increase of *PI3K* and *AKT2* protein and kinase levels in primary breast carcinomas (1, 2). To determine activation of *AKT2* in chemoresistance, we have established pTet-on/pTRE2-HA-DN-*AKT2* and -HA-myr-*AKT2* stably-transfected, i.e. doxycyclin inducible, Hela clonal cell lines. Two (clones 6 and 13) of 18 DN-*AKT2* and 1 (clone 27) of 18 myr-*AKT2* clonal lines are not leaky (Fig. 1A and B). After induction with doxycycline (1  $\mu\text{g/ml}$ ), DN-*AKT2* expresses at very high level, blocks EGF-induced kinase activity of 3 isoforms of endogenous *AKT* (Fig. 1C), and inhibits cell growth (36% less than that in uninduced cells, Fig. 1D). These clonal cell lines were treated with commonly used chemotherapeutic drugs, including cisplatin, paclitaxel, etoposide, vincristine, metotrexate, and doxorubicin. Tetrazolium salt microtiter plate assay (CellTiter 96 Cell Proliferation Assay, Promega) revealed that the cells expressing constitutive activated *AKT2* became to resistant to paclitaxel, etoposide, and cisplatin (Fig 1E) whereas DN-*AKT2* sensitized the cells to some chemotherapeutic reagent-induce cell death (Fig. 1F). These data indicate that activation of *AKT2* contributes to chemoresistance.



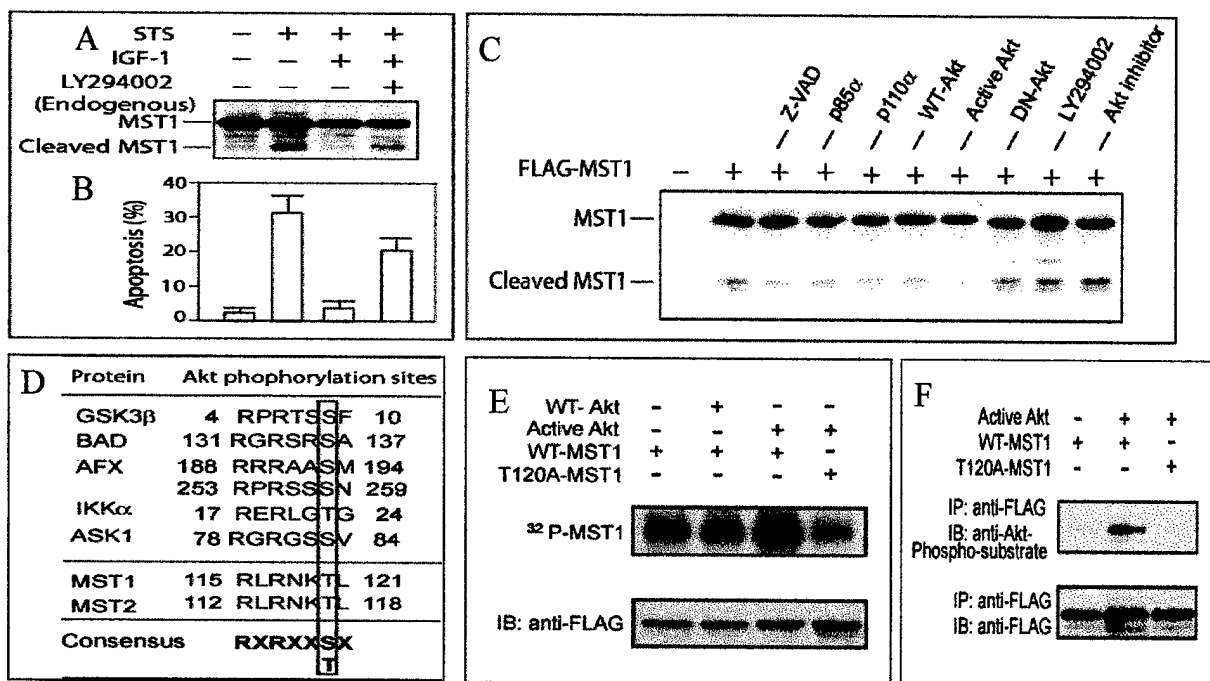
**Fig. 1.** Inducible expression of HA-myr-AKT2 and HA-DN-AKT2 in HeLa-derived cell lines, which are related to chemotherapeutic drug sensitivity. (A) Western blot (top panel) and *in vitro* kinase (bottom panel) analyses of Myr-AKT2 (clones 10 and 27) and DN-AKT2 (clones 6 and 13) clonal cell lines. Cells were treated with (+) or without (-) doxycycline for 48 h. (B) Myr-AKT-27 and DN-AKT-6 cells were exposed to doxycycline for indicated times. Cell extracts were immunoblotted with anti-HA antibody. (C) *In vitro* kinase assay of endogenous AKT1, AKT and AKT3 immunoprecipitates from DN-AKT2-6 cells stimulated with EGF for 15 min after treatment with or without doxycycline (1 µg/ml) for 48 h. (D) Cell growth in DN-AKT2-6 clonal cell line treated with or without doxycycline (1 µg/ml) for indicated times. (E) HeLa cells expressing myr-AKT became resistant to the indicated drugs. (F) HeLa cells expressing DN-AKT are sensitized to cisplatin.

## **2. AKT2 inhibition of cisplatin-induced JNK/p38 activation and Bax conformational change by phosphorylation of ASK1.**

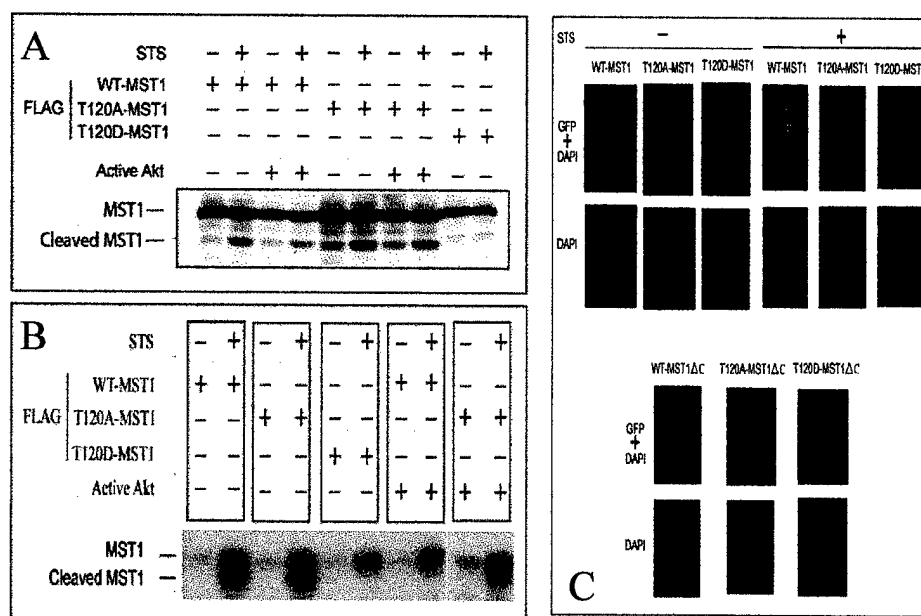
Cisplatin and its analogues have been widely used for treatment of human cancer. However, most patients eventually develop resistance to treatment through a mechanism that remains obscure. Here we demonstrate that constitutively active AKT2 renders cisplatin-sensitive A2780S cells resistant to cisplatin, whereas phosphatidylinositol 3-kinase inhibitor or dominant negative AKT2 sensitizes A2780S and cisplatin-resistant A2780CP cells to cisplatin-induced apoptosis through regulation of the ASK1/JNK/p38 pathway. AKT2 interacts with and phosphorylates ASK1 at Ser-83 resulting in inhibition of its kinase activity. Accordingly, activated AKT2 blocked signaling down-stream of ASK1, including activation of JNK and p38 and the conversion of Bax to its active conformation. Expression of nonphosphorylatable ASK1-S83A overrode the AKT2-inhibited JNK/p38 activity and Bax conformational changes, whereas phosphomimic ASK1-S83D inhibited the effects of cisplatin on JNK/p38 and Bax. Cisplatin-induced Bax conformation change was inhibited by inhibitors or dominant negative forms of JNK and p38. In conclusion, our data indicate that AKT2 inhibits cisplatin-induced JNK/p38 and Bax activation through phosphorylation of ASK1 and thus, plays an important role in chemoresistance. Further, regulation of the ASK1/JNK/p38/Bax pathway by AKT2 provides a new mechanism contributing to its antiapoptotic effects (3).

## **3. Akt negatively regulates MST1 and protects cells from MST1-induced apoptosis.**

The serine/threonine kinase MST1 (mammalian STE20-like kinase 1) can be activated after cleavage by caspases and promotes apoptosis (4), however, the regulation of MST1 has not been well documented. In this study, we demonstrated that MST1 is phosphorylated by Akt at Thr 120 (Fig 2). The phosphorylation leads to the inhibition of cleavage and kinase activity of MST1 at the basal level or under treatment of Staurosporine (STS; Fig 3). Ectopic expression of constitutively active Akt inhibits the cleavage and kinase activity of MST1 induced by STS, however, nonphosphorylatable MST1-T120A (mutation of threonine-120 to alanine) block Akt inhibitory function whereas phosphomimic MST1-T120D (mutation of threonine-120 to aspartic acid) becomes resistant to caspases cleavage and exhibits lower kinase activity. The activation of JNK, a downstream target of MST1, is inhibited in the cells expressing MST1-T120D, but not MST1-T120A. Moreover, the phosphorylated MST1 has the lowest caspase activity and JNK activation (Fig 4). Finally, constitutively active Akt block the nuclear translocation of MST1 (Fig 3C).



**Fig. 2. MST1 is a substrate of Akt.** (A and B) IGF-1 inhibits MST1 cleavage STS-induced cell death.. (C) PI3K and constitutively active Akt inhibit MST1 cleavage, whereas dominant negative Akt, LY294002 and Akt inhibitor enhance MST1 cleavage. (D) Comparison of the putative Akt phosphorylation sites in MST1 with the sequences of phosphorylation sites of known Akt substrates. (E) In vivo labeling (top). Akt phosphorylates MST1 in vivo. Expression of Flag-MST1 was shown in bottom panel. (F) Immunoblotting analysis of the Flag-MST1 immunoprecipitates with anti-Akt-phospho-substrate antibody (top). Bottom panel shows expression of Flag-MST1.



**Fig. 3. Akt phosphorylation of MST1 inhibits cleavage, kinase activity and nuclear localization of MST1.** (A) Akt inhibits STS-induced cleavage and kinase activity of MST1. (B) Phosphomimic MST1-T120D exhibits low levels of kinase activity. (C) GFP-tagged WT and nonphosphorylatable MST1 are localized in the nucleus, whereas phosphomimic MST1 stays in cytosol even following treatment with STS.

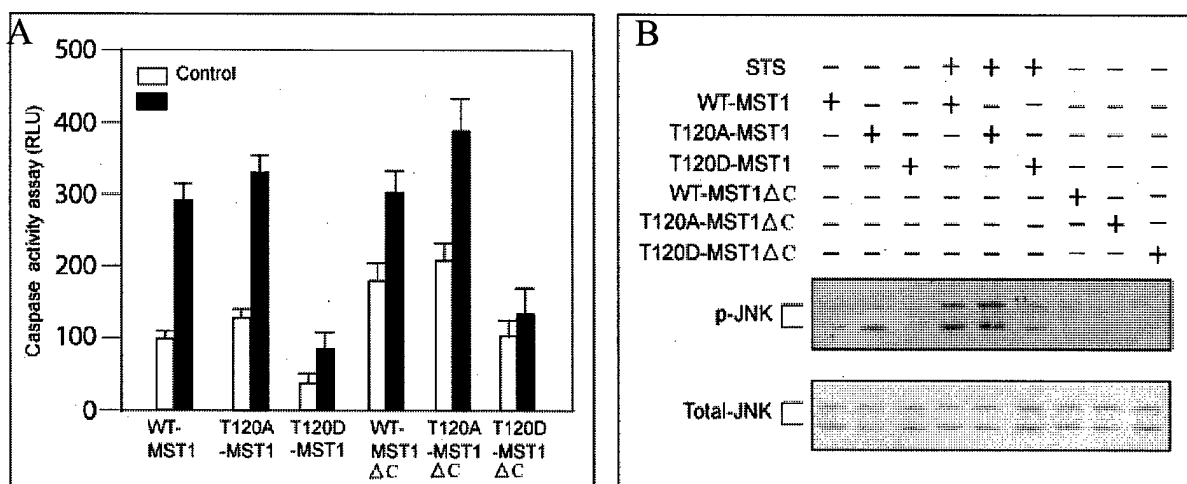


Fig. 4. Akt phosphorylation of MST1 inhibits MST1-induced caspase activity (A) and JNK activation (B).

#### 4. Geranylgeranyltransferase I Inhibitor-298 inhibits AKT2 and survivin pathway to overcome chemoresistance.

GGTI represents a new class of anti-cancer drugs that show promise in blocking the tumor growth (5). However, the mechanism by which GGITs contribute to inhibit tumor cell proliferation is still unclear. We have recently demonstrated that GGTI-298 induces apoptosis in both cisplatin sensitive and resistant human cancer cells by inhibition of PI3K/Akt, including AKT1 and AKT2, and survivin pathways. Following GGTI-298 or GGTI-2166 treatment, kinase levels of PI3K and AKT were decreased and survivin expression was significantly reduced. Ectopic expression of constitutively active AKT2 and/or survivin significantly rescue human cancer cells from GGTI-298-induced apoptosis. Previous studies have shown that Akt mediates growth factor-induced survivin, whereas p53 inhibits survivin expression. However, constitutively active AKT2 failed to rescue the GGITs downregulation of survivin. Further, GGITs suppress survivin expression and induce programmed cell death in both wild type p53 and p53-deficient ovarian cancer cell lines. These data indicate that GGTI-298 and GGTI-2166 induce apoptosis by targeting PI3K/AKT and survivin parallel pathways independent of p53. Due to the fact that upregulation of Akt and survivin as well as inactivation of p53 are frequently associated with chemoresistance (6-8), GGTI could be valuable agents to overcome anti-tumor drug resistance.

#### 5. Create mouse mammary tumor virus (MMTV)-AKT2 transgenic mouse

We have created 3 lines of mouse mammary tumor virus (MMTV) promoter driven-AKT2 transgenic mice, which have been observed for more than 11 months. No breast tumors have developed so far. We plan to maintain the mice for 1.5 years. We will also cross MMTV-AKT2 mice with MMTV-c-erbB2 mice, which will be obtained from Dr. William Muller at Ontario Cancer Institute.

## **Key Research Accomplishment**

- 1 Activation of AKT2 contributes to chemoresistance.
- 2 GGTI inhibits AKT and survivin pathway and overcome cisplatin resistance..
- 3 AKT2 targets ASK1 and MST1 to induce cell survival.

## **Reportable Outcomes**

Dan HC, Coppola D, Jinag K, Liu A, Hamilton AD, Nicosia SV, Sebti SM, **Cheng JQ**. Phosphatidylinositol-3-OH Kinase/Akt and Survivin Pathways as Critical Targets for Geranylgeranyltransferase I Inhibitors Induced Apoptosis. *Oncogene, In press*

Leung BM, Fraser M, Yan X, Dan HC, **Cheng JQ**, Tsang BK. Akt and p53 Are Determinants of Xiap-Mediated Chemoresistance in Human Ovarian Cancer. *Cancer Res., In press*

Sun M, Yang L, Feldman RI, Sun X, Jove R, Nicosia SV, **Cheng JQ**. Activation of Phosphatidylinositol-3-OH Kinase/Akt Pathway by Androgen through interaction of p85 $\alpha$ , AR and Src. *J. Biol. Chem. In press*

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Dan H.C., Sun M., Yang L., Feldman R.I., Sui X-M., Yeung R.S., Halley D.J.J., Nicosia S.V., Pledger W.J., **Cheng, J.Q.** PI3K/AKT pathway regulates TSC tumor suppressor complex by phosphorylation of tuberlin. *J. Biol. Chem.* 277:35364-35370, 2002.

## **Conclusion**

1. Activation of PI3K/AKT2 pathway rendered cells resistant to chemotherapeutic drugs.
2. ASK1 and MST1 are physical substrates of AKT.
3. AKT induces cell survival by inhibition of ASK1 and MST1.
4. GGTI inhibition of AKT and survivin pathway to induce apoptosis.



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## Appendices

1. Yuan Z, Feldman RI, Sussman GE, Coppola D, Nicosia SV, **Cheng JQ.** AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: Implication of AKT2 in chemoresistance. *J. Biol. Chem.* 278:23432-13440, 2003.
2. Dan H.C., Sun M., Yang L., Feldman R.I., Sui X-M., Yeung R.S., Halley D.J.J., Nicosia S.V., Pledger W.J., **Cheng, J.Q.** PI3K/AKT pathway regulates TSC tumor suppressor complex by phosphorylation of tuberlin. *J. Biol. Chem.* 277:35364-35370, 2002.

## AKT2 Inhibition of Cisplatin-induced JNK/p38 and Bax Activation by Phosphorylation of ASK1

IMPLICATION OF AKT2 IN CHEMORESISTANCE\*

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Cisplatin and its analogues have been widely used for treatment of human cancer. However, most patients eventually develop resistance to treatment through a mechanism that remains obscure. Previously, we found that AKT2 is frequently overexpressed and/or activated in human ovarian and breast cancers. Here we demonstrate that constitutively active AKT2 renders cisplatin-sensitive A2780S ovarian cancer cells resistant to cisplatin, whereas phosphatidylinositol 3-kinase inhibitor or dominant negative AKT2 sensitizes A2780S and cisplatin-resistant A2780CP cells to cisplatin-induced apoptosis through regulation of the ASK1/JNK/p38 pathway. AKT2 interacts with and phosphorylates ASK1 at Ser-83 resulting in inhibition of its kinase activity. Accordingly, activated AKT2 blocked signaling downstream of ASK1, including activation of JNK and p38 and the conversion of Bax to its active conformation. Expression of nonphosphorylatable ASK1-S83A overrode the AKT2-inhibited JNK/p38 activity and Bax conformational changes, whereas phosphomimic ASK1-S83D inhibited the effects of cisplatin on JNK/p38 and Bax. Cisplatin-induced Bax conformation change was inhibited by inhibitors or dominant negative forms of JNK and p38. In conclusion, our data indicate that AKT2 inhibits cisplatin-induced JNK/p38 and Bax activation through phosphorylation of ASK1 and thus, plays an important role in chemoresistance. Further, regulation of the ASK1/JNK/p38/Bax pathway by AKT2 provides a new mechanism contributing to its antiapoptotic effects.

Although cisplatin and its analogues, the DNA cross-linking agents, are first-line chemotherapeutic agents for the treatment of human ovarian and breast cancers, chemoresistance remains a major hurdle to successful therapy (1, 2). Several molecules have been implicated in cisplatin resistance, includ-

ing decreased cellular detoxication (3, 4), increased DNA repair (5), and mutations of *p53* tumor suppressor gene (6, 7). However, the mechanisms involved in cisplatin resistance are still poorly understood. A growing body of evidence indicates that defects in the intra- and extracellular survival/apoptotic pathways are an important cause of resistance to cytotoxic agents.

Phosphatidylinositol 3-kinase (PI3K)/Akt is a major cell survival pathway that has been extensively studied recently (8). PI3K is a heterodimer composed of a p85 regulatory and a p110 catalytic subunit and converts the plasma membrane lipid phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. Pleckstrin homology domain-containing proteins, including Akt, accumulate at sites of PI3K activation by directly binding to phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. Akt (also known as PKB) represents a subfamily of serine/threonine kinases. Three member of this family, including AKT1, AKT2, and AKT3, have been identified so far. Akt is activated in a PI3K-dependent manner by a variety of stimuli, including growth factors, protein phosphatase inhibitors, and stress (9–12). Downstream targets of Akt contain the consensus phosphorylation sequence RXX(X)(S/T)(F/L) (13). Several targets of Akt that have been identified have roles in the regulation of apoptosis, such as the proapoptotic proteins BAD and caspase-9 and transcription factor FKHR1. Phosphorylation by Akt blocks BAD binding to Bcl-x<sub>L</sub>, inhibits caspase-9 protease activity, and blocks FKHR1 function, reducing Fas ligand transcription (14–16).

Among Akt family members, AKT2 has been shown to be predominantly involved in human malignancies including ovarian cancer. We have demonstrated previously amplification of the AKT2 in a number of human ovarian cancer cell lines and recently detected frequently elevated protein and kinase levels of AKT2 in about a half of primary ovarian carcinoma examined (17, 18). Moreover, ectopic expression of wild type of AKT2 but not Akt1 in NIH 3T3 cells resulted in malignant transformation (19). Inhibition of PI3K/AKT2 by farnesyltransferase inhibitor-277 induced apoptosis in ovarian cancer cells that overexpress AKT2 (20). We have also shown that TNF $\alpha$  and extracellular stresses, including UV irradiation, heat shock, and hyperosmolarity, induce AKT2 kinase and that

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<sup>1</sup> The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; ASK1, apoptosis signal-regulating kinase 1; JNK, c-Jun NH<sub>2</sub>-terminal kinase; PARP, poly(ADP-ribose) polymerase; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; GST, glutathione S-transferase; HEK, human embryonic kidney; MKK, mitogen-activated protein kinase kinase.

activated AKT2 inhibits JNK/p38 activity to protect cells from TNF $\alpha$  and cellular stress-induced apoptosis (21).

JNK and p38 are predominantly activated through environmental stresses, including osmotic shock, UV radiation, heat shock, oxidative stress, protein synthesis inhibitors, stimulation of Fas, and inflammatory cytokines such as TNF $\alpha$  and interleukin-1. Stimulation of JNK/p38 activity has also been shown to be critical for cisplatin-induced apoptosis in some cancer cells (22, 23). Specific inhibition of JNK or p38, through small molecule inhibitors, dominant negative JNK/p38 mutants, or knock-out of JNK expression, suppresses various types of stress-induced apoptosis (24). Although it has been shown that JNK phosphorylates and inhibits antiapoptotic protein Bcl-2 (25), the mechanism of JNK/p38 induction of apoptosis is still not well understood.

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated protein kinase kinase family that activates both the SEK1-JNK and MKK3/MKK6-p38 signaling cascades (26–28). ASK1 is a general mediator of cell death in response to a variety of stimuli, including oxidative stress (29, 30) and chemotherapeutic drugs such as cisplatin and paclitaxel (22, 23). Ectopic expression of ASK1 induced apoptosis in various cell types (26, 28). Furthermore, disruption of the ASK1 gene in mice causes a remarkable reduction in sensitivity to stress-induced cell death, such as that promoted by TNF $\alpha$  or oxidative stress (33). These data indicate that ASK1 plays a key proapoptotic function through promoting the sustained activation of JNK/p38 mitogen-activated protein kinases.

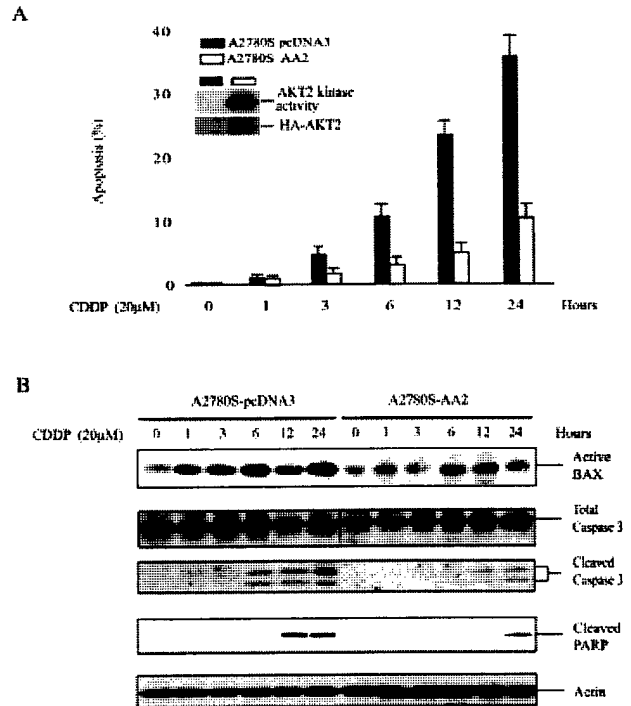
In the present study, we show that AKT2 activity promotes resistance to cisplatin-induced apoptosis in A2780S ovarian cancer cells through the inhibition of the ASK1/JNK/p38 pathway. In A2780S cells, we show that AKT2 complexes with and phosphorylates ASK1 at Ser-83 within a consensus Akt phosphorylation site on this molecule. This results in inhibition of ASK1 activity and the blocking of JNK and p38 activation. We also show that these latter activities are required for cisplatin-induced apoptosis in A2780S cells. Furthermore, in response to cisplatin, we observe that ASK1 and JNK/p38 promote Bax conformational change. Collectively, these studies indicate that AKT2 may be an important mediator of chemoresistance through its regulatory effects on the ASK1/JNK/p38/Bax pathway.

#### EXPERIMENTAL PROCEDURES

**Reagents**—Cisplatin, LY294002, and anti-Bax (6A7) were obtained from Sigma. DMEM and fetal bovine serum were purchased from Invitrogen. Anti-phospho-Akt (Ser-473), anti-cleaved PARP, anti-phospho-JNK (p54/44), anti-phospho-extracellular signal-regulated kinase 1/2 (44/42), anti-phospho-p38, anti-phospho-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2, and anti-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 antibodies were obtained from Cell Signaling (Beverly, MA). GST-c-Jun and GST-ATF6 were also purchased from Cell Signaling. Anti-AKT2, anti-Bax, and anti-ASK1 were obtained from Santa Cruz Biotechnology. JNK inhibitor II and p38 inhibitor SB203580 were from Calbiochem.

**Cell Culture and Cisplatin Treatment**—The human epithelial cancer cell lines, A2780S and A2780CP, kindly provided by Benjamin K. Tsang at The Ottawa Hospital, and human embryonic kidney (HEK) 293 cells were cultured at 37 °C and 5% CO<sub>2</sub> in DMEM supplemented with 10% fetal bovine serum. The cells were seeded in 60-mm Petri dishes at a density of  $0.5 \times 10^6$  cells per dish. After 24 h, cells were treated with cisplatin (20  $\mu$ M) for the appropriate time as noted in the figure legends.

**Expression Constructs**—The cytomegalovirus-based expression constructs encoding wild type HA-AKT2 and constitutively active HA-Myr-AKT2 have been described previously (31). The pcDNA<sub>3</sub>-HA-ASK1 construct was kindly provided by Hidenori Ihijo at Tokyo Medical and Dental University. HA-ASK1-S83A and ASK1-S83D, as well as dominant negative AKT2 with triple mutations (T309A, E299K, and S474A), were created using the QuikChange site-directed mutagenesis kit (Stratagene). JNK and p38 plasmids were obtained from Roger Davis at the University of Massachusetts.

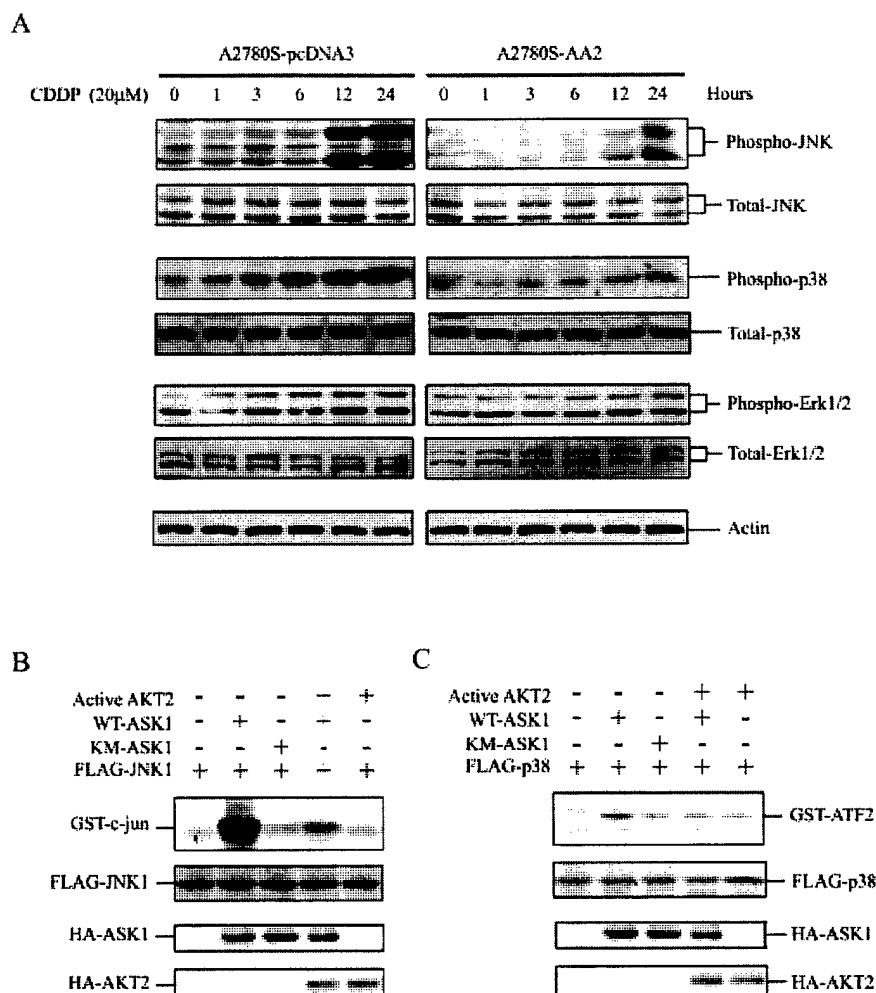


**FIG. 1. Activation of AKT2 renders cells resistant to cisplatin and inhibits cisplatin-induced Bax conformational change and caspase-3 cleavage.** A, cisplatin-sensitive A2780S cells were stably transfected with constitutively active AKT2 (A2780S-AA2) or pcDNA3 (A2780S-pcDNA3). Expression and kinase activity of transfected AKT2 were examined by Western blot and *in vitro* kinase assays (inset). The cells were treated with cisplatin (CDDP; 20  $\mu$ M) for indicated time and analyzed by TUNEL assay. Apoptotic cells were quantified in triple experiments. B, Western blot analysis. The cells were treated with cisplatin and lysed. A portion of lysate was immunoprecipitated with anti-active Bax (6A7) and detected with anti-total Bax antibody (top panel). The rest of the lysates were immunoblotted and probed with anti-caspase-3 (second and third panels), anti-PARP (fourth panel), and anti-actin (bottom panel) antibodies.

**Immunoprecipitation and Immunoblotting**—Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (v/v) glycerol, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 2  $\mu$ g/ml aprotinin and leupeptin, 2 mM benzamide, 20 mM NaF, 10 mM NaPPi, 1 mM sodium vanadate, and 25 mM  $\beta$ -glycerolphosphate. Lysates were centrifuged at 12,000  $\times$  g for 15 min at 4 °C prior to immunoprecipitation or Western blot. Aliquots of the cell lysates were analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1)-agarose beads at 4 °C for 20 min. Following the removal of the beads by centrifugation, lysates were incubated with appropriate antibodies in the presence of 25  $\mu$ l of protein A-protein G (2:1)-agarose beads for at least 2 h at 4 °C. The beads were washed with buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 M LiCl, and 0.5% Triton X-10; twice with phosphate-buffered saline; and once with buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, and 1 mM dithiothreitol, all supplemented with 20 mM  $\beta$ -glycerolphosphate and 0.1 mM sodium vanadate. The immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein expression was determined by probing Western blots of immunoprecipitates or total cell lysates with the appropriate antibodies as noted in the figure legends. Detection of antigen-bound antibody was carried out with the ECL Western blotting analysis system (Amersham Biosciences).

**In Vitro Kinase Assay**—Protein kinase assays were performed as described previously (21). Briefly, reactions were carried out in the presence of 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (PerkinElmer Life Sciences) and 3  $\mu$ M cold ATP in 30  $\mu$ l of buffer containing 20 mM Hepes (pH 7.4), 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, and 1 mM dithiothreitol. 2  $\mu$ g of myelin basic protein was used as the exogenous substrate. After incubation at room temperature for 30 min the reaction was stopped by adding protein loading buffer, and proteins were separated on SDS-PAGE gels. Each

**FIG. 2. AKT2 inhibits JNK and p38 activation induced by cisplatin and ASK1.** A, immunoblotting analysis. Following treatment with cisplatin at indicated times, the cells were lysed and immunoblotted. The blots were detected with indicated antibodies. B and C, *in vitro* kinase assay. HEK293 cells were transfected with the indicated expression plasmids. After 36 h of transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody. The FLAG-JNK and FLAG-p38 immunoprecipitates were subjected to *in vitro* kinase using GST-c-Jun (B) and GST-ATF2 (C) as substrate, respectively (top panel). Expression of the transfected plasmids was shown in the second, third, and fourth panels.



experiment was repeated three times, and the relative amounts of incorporated radioactivity were determined by autoradiography and quantified with a PhosphorImager (Molecular Dynamics).

**In Vivo [<sup>32</sup>P]P<sub>i</sub> Labeling**—HEK293 cells were co-transfected with active AKT2 and HA-tagged ASK1 or pcDNA3 and labeled with [<sup>32</sup>P]P<sub>i</sub> (0.5 mCi/ml) in phosphate- and serum-free DMEM medium for 4 h. Cell lysates were subjected to immunoprecipitation with anti-HA antibody. The immunoprecipitates were separated by 7.5% SDS-PAGE and transferred to membranes. Phosphorylated ASK1 band was visualized by autoradiography. The expression of transfected ASK1 was detected with anti-HA antibody.

**Luciferase Reporter Assay**—Cells were seeded in 6-well plate and transfected with c-Jun or ATF6 reporter plasmid (pGL4-Luc), pSV2-βgal, and different forms (wild type, constitutively active, or dominant negative) of HA-AKT2 together with or without different forms of ASK1 or vector alone. After 36 h of the transfection, luciferase and β-galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix, respectively). Each experiment was repeated three times.

**Tunel Assay**—Cells were seeded into 60-mm dishes and grown in DMEM supplemented with 10% fetal bovine serum for 24 h and treated with 20 μM cisplatin for different times. Apoptosis was determined by Tunel assay using an *in situ* cell death detection kit (Roche Applied Science). These experiments were performed in triplicate.

## RESULTS

**Activation of AKT2 Renders Cisplatin-sensitive Cells Resistant to Cisplatin and Inhibits Cisplatin-induced Bax Conformational Change**—We have shown previously (18, 34) frequent activation of AKT2 kinase in human ovarian and breast cancers. To examine whether activation of AKT2 contributes to

chemoresistance in cancer cells, cisplatin-sensitive A2780S cells were stably transfected with constitutively active AKT2 (A2780S-AA2) or pcDNA3 vector alone. Expression and kinase activity of transfected constitutively active AKT2 were confirmed by Western blot and *in vitro* kinase analysis (Fig. 1A, inset). Following treatment with cisplatin (20 μM) for 0, 1, 3, 6, 12, and 24 h, programmed cell death in A2780S-pcDNA3 and A2780S-AA2 (active AKT2) cells were examined by Tunel assay. The number of apoptotic cells was quantified by counting three different microscopic fields. Three h after treatment, A2780S-pcDNA3 cells begun to undergo apoptosis. By 24 h of treatment, 35% of the cells were apoptotic, which is a similar response reported in the literature for parental A2780S cells (35). However, we observed a distinctly lower percentage of apoptotic cells at the time points 3, 6, 12, and 24 h in A2780S-AA2 cells (Fig. 1A), indicating that activation of AKT2 renders cisplatin-sensitive A2780S cells resistant to cisplatin.

It has been shown that Bax is required for cisplatin-induced apoptosis, *i.e.* cisplatin activates Bax by inducing its N-terminal conformation change and then targeting it to mitochondria resulting in cytochrome c release and activation of apoptotic pathway (36, 37). Thus, we next examined the effects of AKT2 activation on induction of Bax conformational changes by cisplatin. After treatment with cisplatin, A2780S-pcDNA3 and A2780S-AA2 cells were lysed and immunoprecipitated with anti-active Bax (6A7) antibody. The immunoprecipitates were subjected to Western blot analysis with total anti-Bax anti-

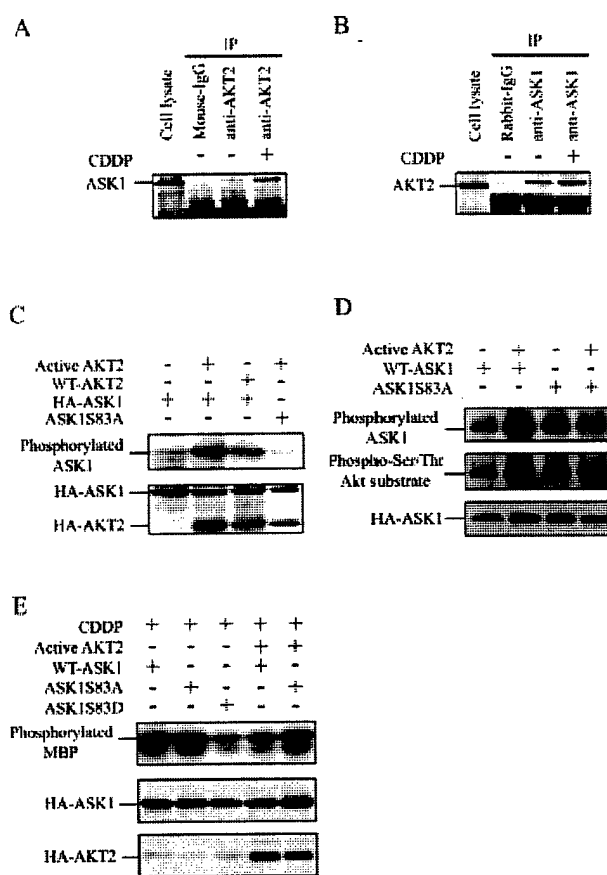
body. As shown in Fig. 1B, cisplatin promotes alteration of Bax conformation after 3 h of treatment in A2780S-pcDNA3 cells but not in A2780S-AA2 cells. Accordingly, cleavage of caspase 3 and its substrate, PARP, was also inhibited by expression of constitutively active AKT2 as compared with pcDNA3-transfected A2780S cells (Fig. 1B).

**AKT2 Inhibits Cisplatin- and ASK1-induced JNK and p38 Activation**—It has been documented that stress kinases, JNK and p38, are activated by cisplatin, and their activations are required for cisplatin-induced programmed cell death (22, 23, 38). To examine whether the effect of cisplatin on JNK and p38 is abrogated by the activation of AKT2, A2780S-pcDNA3 and A2780S-AA2 cells were treated with cisplatin at different times. As expected, JNK and p38 were activated by cisplatin in A2780S-pcDNA3 cells, and the activation of p38 took place before that of JNK. However, the activation of JNK and p38 was reduced dramatically in A2780S cells transfected with a constitutively active AKT2. No significant difference in the phosphorylation levels of extracellular signal-regulated kinase was observed between these two cell lines (Fig. 2A).

To explore the mechanism of AKT2 inhibition of the JNK and p38, we probed for direct interaction of these proteins by co-immunoprecipitation. We were not, however, able to demonstrate any interaction between AKT2 and JNK or p38 (data not shown). As ASK1 is known to activate JNK/p38 and be induced by cisplatin (32), and its overexpression is sufficient to induce apoptosis (26, 28), we next examined whether AKT2 restrains JNK and p38 activity through inhibition of ASK1. HEK293 cells were transfected with FLAG-JNK1 or FLAG-p38 and wild type or kinase-dead ASK1 (KM-ASK1), with or without constitutively active AKT2. After 36 h of transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody. FLAG-JNK1 and FLAG-p38 immunoprecipitates were subjected to *in vitro* kinase assays using GST-c-Jun and GST-ATF2 as substrates, respectively. Repeated experiments revealed that kinase activities of JNK1 and p38 were significantly induced by expression of wild type but not kinase-dead ASK1 and that the activation of JNK and p38 was attenuated by ectopic expression of constitutively active AKT2 (Fig. 2, B and C). These data indicate that AKT2 may negatively regulate ASK1, causing inhibition of cisplatin-induced JNK/p38 activation and apoptosis.

**AKT2 Interacts with, Phosphorylates, and Inhibits ASK1**—To examine whether ASK1 is a direct target of AKT2, co-immunoprecipitation was carried out with anti-AKT2 antibody and detected with anti-ASK1 antibody, and vice versa. As shown in Fig. 3, A and B, interaction between ASK1 and AKT2 was readily detected, and this interaction was enhanced by cisplatin treatment. Sequence analysis revealed that an AKT2 phosphorylation consensus site resides in ASK1 at residue Ser-83, which is conserved between human and mouse. To determine whether AKT2 phosphorylates ASK1, *in vitro* AKT2 kinase assays were performed using immunoprecipitated HA-ASK1 (wild type ASK1 or ASK1S83A) as substrates (Fig. 3C). In addition, *in vivo* [ $^{32}$ P] labeling and immunoblotting analyses with anti-phospho-Ser/Thr Akt substrate antibody were carried out in HEK293 cells transfected with ASK1 and constitutively active or wild type AKT2 (Fig. 3D). Both *in vitro* kinase and *in vivo* labeling experiments, as well as Western blot analysis, showed that wild type and constitutively active AKT2 phosphorylate ASK1 at Ser-83 with the lower phosphorylation level by wild type AKT2 (Fig. 3, C and D).

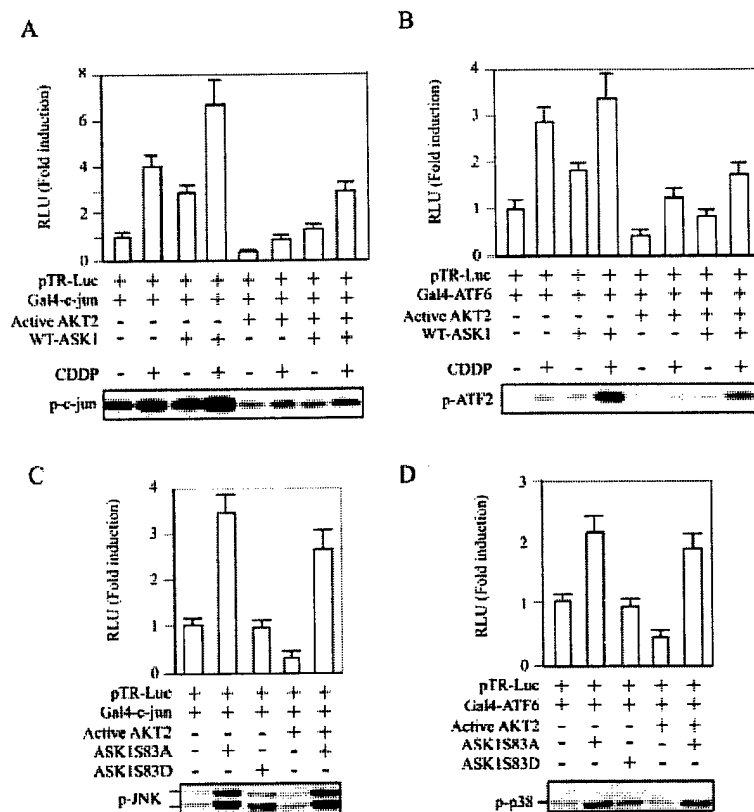
We next determined whether cisplatin-induced ASK1 activation is inhibited by AKT2 and, if it is, whether this inhibition depends upon AKT2 phosphorylation of ASK1 at Ser-83. Mutagenesis was used to create a form of ASK1 not phosphorylat-



**FIG. 3. AKT2 interacts with and phosphorylates ASK1 and inhibits ASK1 kinase activity.** A and B, Western blot analyses of the immunoprecipitates prepared from A2780S cells treated with or without cisplatin. Immunoprecipitation was performed with anti-AKT2 and detected with anti-ASK1 antibody (A) and vice versa (B). C, *in vitro* kinase analysis of AKT2 immunoprecipitates derived from HEK293 cells that were transfected with indicated plasmids. Immunoprecipitated HA-ASK1 or HA-ASK1-S83A was used as substrate (top panel). The bottom panel shows expression of transfected plasmids. D, *in vivo* [ $^{32}$ P] labeling. HEK293 cells were transfected with indicated expression constructs, labeled with [ $^{32}$ P]P<sub>i</sub> (0.5 mCi/ml), and immunoprecipitated with anti-HA antibody. The HA-ASK1 immunoprecipitates were separated in SDS-PAGE, blotted, and exposed to x-ray film (top panel). The membrane was then detected with anti-Akt substrate antibody (middle panel) and anti-HA antibody (bottom panel). E, *in vitro* ASK1 kinase analysis of the immunoprecipitates prepared from A2780S cells transfected with indicated plasmids and treated with cisplatin (20  $\mu$ M) for 6 h. Myelin basic protein was used as substrate (top panel). Expression of transfected different forms of ASK1 and AKT2 was shown in the second and third panels.

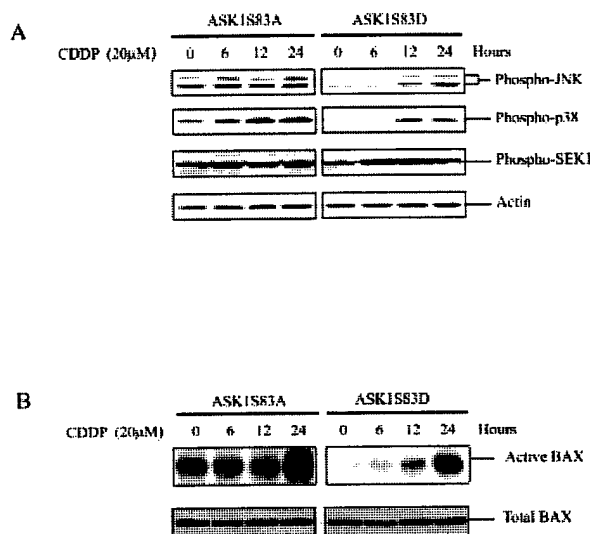
able by AKT2, ASK1-S83A, prepared by converting Ser-83 of ASK1 to alanine. We also prepared ASK1-S83D, derived from mutating Ser-83 of ASK1 to aspartic acid, which mimics ASK1 phosphorylated by AKT2. A2780S cells were transfected with ASK1-S83A or ASK1-S83D, with or without constitutively active AKT2. Following cisplatin treatment, ASK1s were immunoprecipitated, and *in vitro* ASK1 kinase assays were performed using myelin basic protein as substrate. As shown in Fig. 3E, cisplatin significantly induced the kinase activity of both wild type ASK1 and nonphosphorylatable ASK1-S83A but not AKT2 phosphomimic ASK1-S83D. Expression of constitutively active AKT2 inhibited cisplatin-stimulated kinase activity of wild type ASK1 but not that of nonphosphorylatable ASK1-S83A. These results indicate that ASK1 kinase activity is negatively regulated by AKT2 through phosphorylation of ASK1 at Ser-83.

**FIG. 4. Activation of AKT2 inhibits ASK1- and/or cisplatin-induced JNK and p38 activation.** A–D, luciferase reporter assays. A2780S cells were transfected with indicated expression constructs and treated with or without cisplatin. Luciferase and  $\beta$ -galactosidase assays were performed, and the reporter activity was normalized by dividing luciferase activity with  $\beta$ -galactosidase. Each experiment was repeated three times. The bottom panels of A and B show the results obtained from *in vitro* JNK and p38 kinase assays using GST-c-Jun and ATF2 as substrates, respectively. The effects of AKT2 and its phosphorylation of ASK1 at Ser-83 on JNK and p38 activation were shown in the bottom panels of C and D.



**AKT2 Inhibition of Cisplatin-stimulated JNK and p38 Is Mediated by Phosphorylation of ASK1 at Residue Ser-83**—We next determined whether phosphorylation of ASK1 on Ser-83 by AKT2 is required for AKT2 inhibition of p38 and JNK, which are downstream from ASK1. Luciferase reporter assays were performed using Gal4-c-Jun/pTR-Luc (for JNK) and Gal4-ATF6/pTR-Luc (for p38) reporter systems. A2780S cells were transfected with ASK1, ASK1-S83A, ASK1-S83D, and/or Myr-AKT2, as well as pTR-Luc, Gal4-c-Jun, or Gal4-ATF6, and treated with or without cisplatin. Three independent experiments revealed that cisplatin induces Gal4-c-Jun or Gal4-ATF6-regulated reporter activities. Further, *in vitro* JNK and p38 kinase analysis revealed that the phosphorylation of c-Jun and ATF2 was also stimulated by cisplatin treatment. These effects were enhanced by ectopic expression of wild type ASK1; however, they were inhibited by expression of constitutively active AKT2 (Fig. 4, A and B). Expression of nonphosphorylatable ASK1-S83A was also sufficient to induce the reporter activities and to attenuate the inhibitory action of constitutively active AKT2. In contrast, phosphomimic ASK1-S83D failed to stimulate the reporter activities (Fig. 4, C and D). Moreover, the effects of ASK1-S83A and ASK1-S83D on cisplatin-induced JNK and p38 activation were similar to their action on Gal4-c-Jun and Gal4-ATF6 reporters (Fig. 5A). Therefore, we conclude that AKT2 inhibits cisplatin-induced JNK and p38 via a phosphorylation of ASK1-dependent manner.

**Cisplatin-induced Bax Conformational Change Is Regulated by AKT2 Phosphorylation of ASK1**—Previous studies have shown that JNK is required for UV- and cisplatin-induced Bax conformational change (39). Our data demonstrate that ectopic expression of constitutively active AKT2 overrides cisplatin-induced ASK1/JNK/p38 activation and prevents formation of the active Bax conformation (see Figs. 1 and 2). To more directly probe the effect of AKT2 phosphorylation of ASK1 on Bax activation, we transfected A2780S cells with nonphosphorylat-



**FIG. 5. AKT2 phosphorylation of ASK1 at Ser-83 plays a critical role in cisplatin-induced JNK/p38 activation and Bax conformational change.** A, immunoblotting analysis of A2780S cells transfected with nonphosphorylatable and phosphomimic ASK1 prior to treatment with cisplatin. The blots were probed with the indicated antibodies. B, Western blot analysis. A2780S cells were transfected with indicated expression plasmids, treated with cisplatin, immunoprecipitated with anti-active Bax antibody, and detected with anti-total Bax antibody (top panel). Expression of Bax was shown in the bottom panel.

able and phosphomimic ASK1 and treated the cells with or without cisplatin. As revealed by immunoprecipitation and Western blot analyses, ectopic expression of nonphosphorylatable ASK1-S83A enhance cisplatin-dependent Bax conformational change, whereas ASK1-S83D, mimicking ASK1 phosphorylated by AKT2, inhibited cisplatin-induced Bax

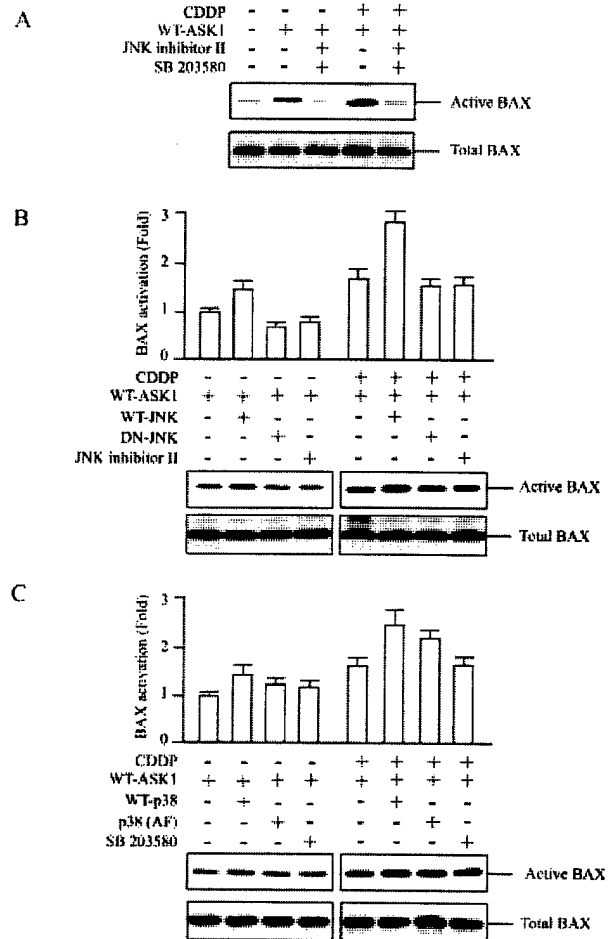
activation (Fig. 5B versus Fig. 1B). These data suggest that AKT2 inhibition of cisplatin-stimulated Bax conformational change is mediated at least to some extent by AKT2 phosphorylation of ASK1 at residue Ser-83.

Because JNK and p38 are downstream targets of ASK1, we next examined their roles in ASK1-stimulated Bax activation by using selective small molecule inhibitors of JNK and p38, JNK inhibitor II and SB 203580. As illustrated in Fig. 6A, expression of ASK1 was sufficient to induce a Bax conformational change, and this effect was enhanced by cisplatin treatment. However, the conformational change of Bax induced by ASK1 and/or cisplatin was significantly diminished following treatment of cells with JNK inhibitor II (10  $\mu$ M) and p38 inhibitor, SB 203580 (10  $\mu$ M), suggesting that JNK and/or p38 mediate cisplatin-induced Bax activation. To probe the individual contributions of JNK and p38 in cisplatin-stimulated Bax activation, we further examined the effects of small molecule inhibitors of p38 and JNK and the expression of wild type and dominant negative forms of these kinases. A2780S cells were transfected with wild type or dominant negative JNK or p38, together with ASK1, and treated with or without cisplatin and/or inhibitor of JNK or p38. As shown in Fig. 6, B and C, expression of wild type JNK or p38 enhanced ASK1- and cisplatin-induced Bax activation, as expected. Furthermore, dominant negative JNK or a small molecule JNK inhibitor significantly decreased the Bax activation induced by cisplatin treatment or ectopic expression of ASK1 (Fig. 6B). We observed that only slight inhibition of the Bax activation was in the cells expressing dominant negative p38 or treated with p38 inhibitor (Fig. 6C). These results indicate that cisplatin- and/or ASK1-induced Bax activation is mediated primarily by JNK.

**Inhibition of PI3K/AKT2 Pathway Sensitizes Cells to Cisplatin-induced Apoptosis**—Because activated AKT2 reduces the cisplatin sensitivity of A2780S cells, we next examined the ability of inhibition of the PI3K/AKT2 pathway to sensitize cells to cisplatin-induced apoptosis. Cisplatin-resistant A2780CP and A2780S cells were transfected with dominant negative AKT2 or treated with PI3K inhibitor, LY294002, together with cisplatin. TUNEL assay analyses revealed that either LY294002 or ectopic expression of dominant negative AKT2 enhanced cisplatin-induced apoptosis as compared with cells treated with cisplatin alone (Fig. 7, A and C). Accordingly, cleavage of caspase-3 and PARP was increased by treatment of cells with a combination of cisplatin with LY294002 or dominant negative-AKT2 (Fig. 7, B and D). To examine the role played by AKT2 phosphorylation of ASK1 in cisplatin-induced apoptosis, we transfected A2780S cells with ASK1-S83A, which is not phosphorylated by AKT2, ASK1-S83D, which mimics AKT2 phosphorylation, and then induced apoptosis with cisplatin. Notably, ectopic expression of ASK1-S83A significantly augmented cisplatin-induced apoptosis. In contrast, expression of ASK1-S83D conferred resistance to cisplatin (Fig. 7E). These data further indicate that PI3K/AKT2 promotes cell survival through phosphorylation and inhibition of ASK1 signaling.

#### DISCUSSION

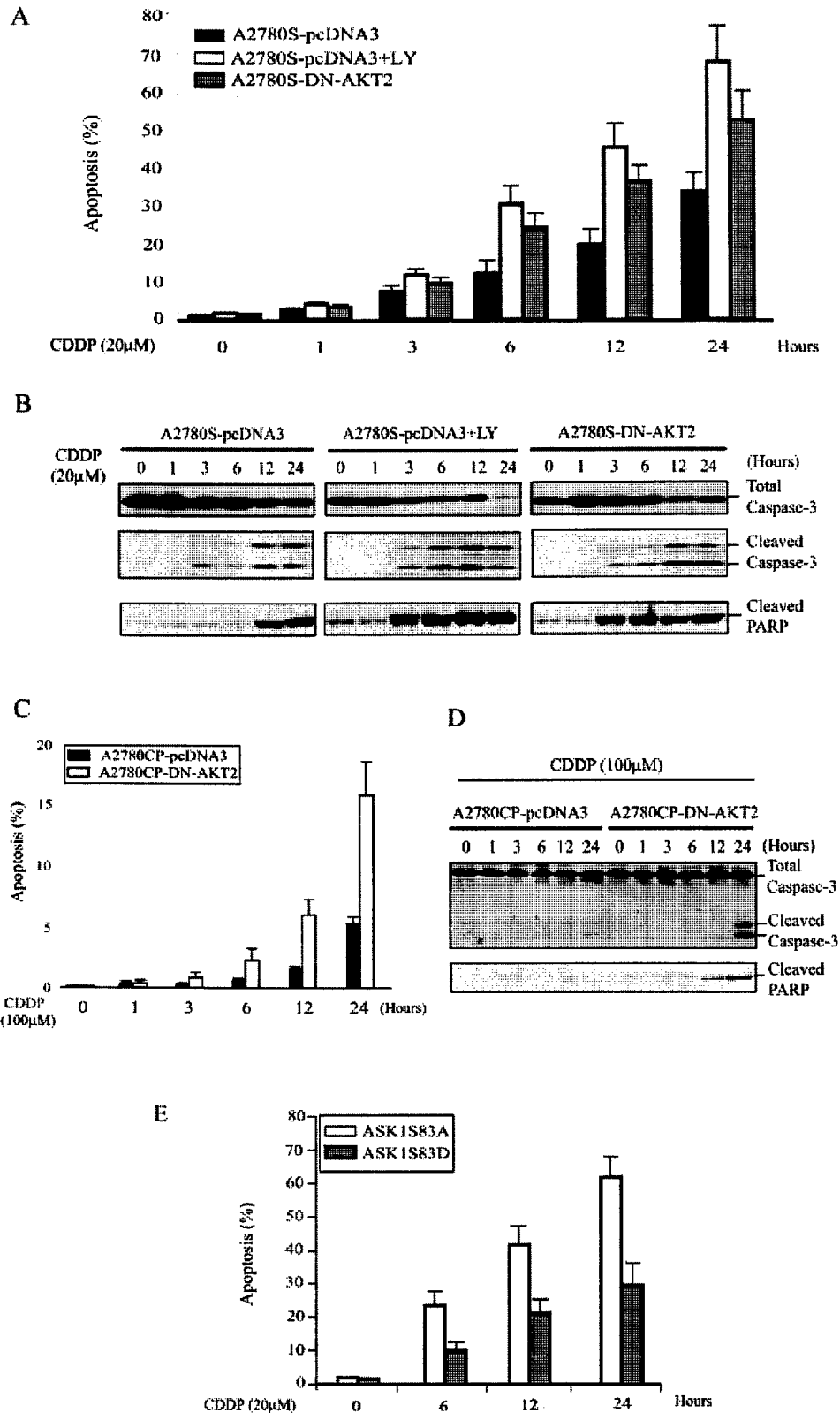
We have demonstrated previously (18, 34) that AKT2 kinase is frequently elevated in human ovarian and breast cancers and that AKT2, like Akt1, exerts its anti-apoptotic function through phosphorylation of Bad (20). However, the biological role of AKT2 activation in human cancer and the mechanism of AKT2-induced cell survival in a chemotherapeutic setting have not been well documented. In this study, we show that activation of AKT2 significantly increases the resistance of ovarian cancer cells to cisplatin. AKT2 protects cells from cisplatin-induced apoptosis by inhibiting cisplatin-induced JNK/p38 activation and Bax conformational change.



**FIG. 6. JNK and p38 mediate cisplatin- and ASK1-induced Bax conformational change.** A, Western blot analysis. A2780S cells were transfected with ASK1 and treated with JNK inhibitor II (10  $\mu$ M) and SB 203580 (10  $\mu$ M) for 1 h prior to addition of cisplatin. Following 16 h of the further treatment, Bax conformational change was examined as described above. B and C, immunoblotting analyses. A2780S cells were transfected with indicated plasmids and treated with indicated reagents. Bax conformational change was evaluated as described above. Both JNK inhibitor and dominant negative JNK exhibited more significant inhibitory effects on Bax activation than did p38 inhibitor and dominant negative p38 (AF). All the experiment was repeated three times.

AKT2 mediates these effects through its interaction and phosphorylation of ASK1.

Cisplatin-induced JNK and p38 activations are required for its anti-tumor activity (22, 23). This activation has been shown to correlate with induction of apoptosis by cisplatin (22, 23). Moreover, studies using dominant negative mutants of JNK and p38 and specific pharmacological inhibitors have shown that activation of JNK and/or p38 is necessary for stress or chemotherapeutic drug-induced apoptosis (38, 40). Also, studies on fibroblasts with targeted disruptions of all the functional *Jnk* genes established an essential role for JNK in UV- and other stress-induced apoptosis (41). ASK1, an upstream regulator of JNK/p38, has also been shown to be induced by cisplatin (32). Furthermore, oxidative stress-induced ASK1 kinase activity is inhibited by Akt1 (42). Consistent with this, we demonstrate that activation of AKT2 inhibits cisplatin-induced JNK and p38 through direct interaction with and phosphorylation of ASK1 at serine 83. We also demonstrate that phosphorylation of ASK1 by AKT2 renders cells resistant to cisplatin.



**FIG. 7. Inhibitions of PI3K/AKT2 and ASK1 phosphorylation sensitize cells to cisplatin-induced apoptosis.** *A*, TUNEL assay. A2780S cells were transfected with dominant negative AKT2 or pcDNA3 vector and treated with cisplatin or cisplatin/LY294002. Apoptosis was examined and quantified after treatment for the indicated times. *B*, immunoblotting analysis of cell lysates prepared from cells treated as *A*. The blots were probed with indicated antibodies. *C* and *D*, cisplatin-resistant A2780CP cells transfected, treated, and analyzed as described in *A* and *B* except LY294002 treatment. *E*, TUNEL assay. A2780S cells were transfected with indicated plasmids and treated with cisplatin. All the experiments were performed in triplicate.



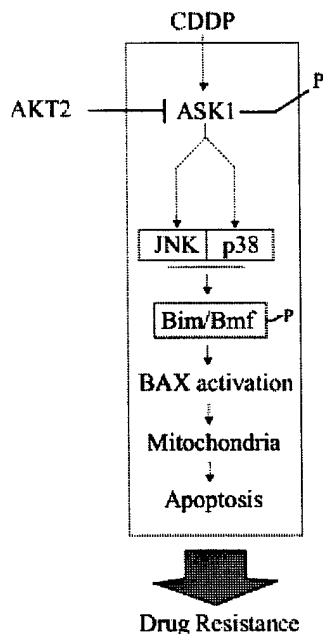


FIG. 8. Schematic illustration of AKT2 regulation of ASK1/JNK/p38 and Bax.

Besides the direct inhibition of ASK1, AKT2 could regulate JNK and p38 through other mechanisms. For example, NF $\kappa$ B-induced X chromosome-linked inhibitor of apoptosis and GADD45 $\beta$  down-regulate TNF $\alpha$ -induced JNK signaling (43, 44). We have demonstrated previously (21) that AKT2 inhibits UV- and TNF $\alpha$ -induced JNK and p38 by activation of the NF $\kappa$ B pathway (21). Therefore, we examined the possibility of AKT2 up-regulation of X chromosome-linked inhibitor of apoptosis and GADD45 $\beta$ . Western and Northern blot analyses, however, revealed no difference in X chromosome-linked inhibitor of apoptosis and GADD45 $\beta$  expression in A2780S cells transfected with constitutively active AKT2 or the control plasmid, pcDNA3 (data not shown). The possible reason is that cisplatin, unlike UV and TNF $\alpha$ , is incapable of inducing the NF $\kappa$ B pathway in A2780S cells. In fact, our reporter assay revealed that cisplatin inhibits rather than activates NF $\kappa$ B activity in A2780S cells (data not shown).

In the present study, we observed that the ability of AKT2 to inhibit cisplatin-induced JNK/p38 was attenuated by nonphosphorylatable ASK1-S83A. Expression of phosphomimic ASK1-S83D alone was sufficient to inhibit JNK/p38 activation (Fig. 4). In addition, ASK1-S83D exhibited effects similar to that of constitutively active AKT2, *i.e.* rendered cells resistant to cisplatin, whereas ASK1-S83A sensitized cells to cisplatin-induced apoptosis (Fig. 7E). Thus, we conclude that AKT2 inhibition of cisplatin-stimulated JNK/p38 activation leading to cisplatin resistance is mediated by AKT2 phosphorylation/inhibition of ASK1.

It has been demonstrated that cisplatin-induced Bax conformational change is also important for cisplatin-stimulated apoptosis (45). Bax is a pro-apoptotic member of the Bcl2 family. Accumulated evidence shows that death signals, including cisplatin, induce a conformational change of Bax, leading to its mitochondrial translocation, oligomerization or cluster formation, and cytochrome *c* release (46, 47). Recent studies from Bax and/or Bak knock-out cells have shown that BH3-only proteins, such as tBid, Bad, Puma, and Bim, are required for inducing the activation of Bax and Bak by their direct interaction (48). Moreover, Akt has been shown to effectively inhibit Bax conformational change and contribute to chemoresistance (49).

However, the mechanism by which Akt blocks Bax activation is poorly documented. We demonstrate in this report that ASK1 mediates at least in part cisplatin-induced Bax conformational change. Ectopic expression of constitutively active AKT2 attenuates cisplatin-induced Bax activation by phosphorylation and inhibition of ASK1. Downstream targets of ASK1, JNK, and p38, especially JNK, mediate AKT2 inhibition of Bax conformational change. These results are consistent with the recent findings obtained from a *Jnk*-deficient cell model (39).

Accumulated evidence shows that AKT2 plays a more significant role in human oncogenesis than AKT1 and AKT3. Frequent alterations of AKT2, but not AKT1 and AKT3, were detected in human cancers (18). Further, ectopic expression of AKT2, but not AKT1 and AKT3, leads to increased invasion and metastasis of human breast and ovarian cancer cells (50) and to malignant transformation of mouse fibroblasts (19). We observed in this study that A2780S cells expressing constitutively active AKT2 became cisplatin-resistant whereas expression of dominant negative AKT2 or treatment with PI3K inhibitor sensitized both cisplatin-sensitive (A2780S) and -resistant (A2780CP) ovarian cancer cells to cisplatin-induced apoptosis. Moreover, cisplatin-induced programmed cell death was enhanced by the expression of AKT2 nonphosphorylatable ASK1-S83A, whereas it is inhibited by phosphomimic ASK1-S83D. These data, therefore, indicate that activation of AKT2 contributes to cisplatin resistance by regulation of the ASK1/JNK/p38/Bax pathway and that the PI3K/AKT2/ASK1 cascade could be a critical therapeutic target for human cancer (Fig. 8).

A recent report (51) demonstrates that JNK and p38 phosphorylate BH3-only proapoptotic proteins Bim and Bmf, which was thought to mediate UV-induced apoptosis through a Bax-dependent mitochondrial apoptotic pathway (Fig. 8). Further investigation is required to determine the molecular mechanism by which ASK1/JNK/p38 regulates Bax activation in ovarian cancer cells, *i.e.* whether ASK1 and/or cisplatin induce Bim and Bmf phosphorylation and whether the phosphorylation is inhibited by PI3K/AKT2 pathway.

**Acknowledgments**—We are grateful to Hidenori Ihijo for pcDNA3-HA-ASK1, Roger Davis for JNK and p38 plasmids, and Benjamin K. Tsang for ovarian cancer cell lines. We are also grateful to the DNA Sequence Facility at H. Lee Moffitt Cancer Center for sequencing ASK1 mutant expression constructs.

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## Phosphatidylinositol 3-Kinase/Akt Pathway Regulates Tuberous Sclerosis Tumor Suppressor Complex by Phosphorylation of Tuberin\*

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Normal cellular functions of hamartin and tuberin, encoded by the *TSC1* and *TSC2* tumor suppressor genes, are closely related to their direct interactions. However, the regulation of the hamartin-tuberin complex in the context of the physiologic role as tumor suppressor genes has not been documented. Here we show that insulin or insulin growth factor (IGF) 1 stimulates phosphorylation of tuberin, which is inhibited by the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 but not by the mitogen-activated protein kinase inhibitor PD98059. Expression of constitutively active PI3K or active Akt, including Akt1 and Akt2, induces tuberin phosphorylation. We further demonstrate that Akt/PKB associates with hamartin-tuberin complexes, promoting phosphorylation of tuberin and increased degradation of hamartin-tuberin complexes. The ability to form complexes, however, is not blocked. Akt also inhibits tuberin-mediated degradation of p27<sup>kip1</sup>, thereby promoting CDK2 activity and cellular proliferation. Our results indicate that tuberin is a direct physiological substrate of Akt and that phosphorylation of tuberin by PI3K/Akt is a major mechanism controlling hamartin-tuberin function.

Tuberous sclerosis complex (TSC)<sup>1</sup> is an autosomal dominant disorder and is characterized by the presence of hamartomas in many organs such as brain, skin, heart, lung, and kidney (1). It is caused by mutation of either the *TSC1* or *TSC2* tumor suppressor gene (2–5). *TSC1* encodes a protein, hamartin, containing two coiled-coil domains that have been shown to mediate binding to hamartin (6). The *TSC2* gene codes for tuberin, which contains a small region of homology to the rap1GTPase-

activating protein, rap1GAP (7). These two proteins function within the same pathway(s) regulating cell cycle, cell growth, adhesion, and vesicular trafficking (4, 5). However, the regulation of hamartin and tuberin in the context of physiologic role as tumor suppressor genes has not been documented.

Among the various properties of these two proteins, the ability to interact and to form stable complex has been the most consistent finding. This led to the hypothesis that hamartin and tuberin function as a complex and that factors regulating their interaction are important in understanding physiologic roles. There is evidence to suggest that phosphorylation of tuberin may be a major mechanism of regulation of the hamartin-tuberin complex (8, 9). However, the kinases that are responsible for phosphorylation of this complex are currently unknown. Recent *Drosophila* genetic studies showed that *dTsc1* and *dTsc2* play an important role in the insulin/dPI3K/dAkt signal transduction pathway by demonstrating that reduced cell size and cell proliferation caused by either mutations in *dINR* and *dAkt* or by overexpression of *dPTEN* are overridden by homozygous mutants of *dTsc1* or *dTsc2*. This implies that *dTsc1* and *dTsc2* are either direct downstream targets of dAkt or on a parallel pathway of the insulin cascade downstream from dAkt (10–13). Akt, also known as protein kinase B (PKB), represents a subfamily of the serine/threonine protein kinase. Three isoforms of Akt have been identified including Akt1/PKB $\alpha$ , Akt2/PKB $\beta$ , and Akt3/PKB $\gamma$ , all of which are activated by growth factors and insulin in a PI3K-dependent manner and are inhibited by PTEN tumor suppressor (14). Akt regulates a wide spectrum of cell functions, including cell survival, cell growth, differentiation, angiogenesis, and glucose metabolism, through phosphorylation of a number of proteins that contain the RXRXXS/T motif (14–16).

Here we show that Akt physically interacts with and phosphorylates tuberin, leading to degradation of the hamartin-tuberin complex and p27<sup>kip1</sup> without interfering with hamartin-tuberin complex formation. Moreover, IGF1 and insulin induce tuberin phosphorylation, which is mediated by the PI3K/Akt pathway but not by the MAPK pathway. As a result, cyclin-dependent kinase (CDK) 2 activity, DNA synthesis, and S phase of the cell cycle are elevated. We thus have identified Akt as a major tuberin kinase to negatively regulate hamartin-tuberin tumor suppressor function by inducing degradation.

### EXPERIMENTAL PROCEDURES

**Plasmids**—The cytomegalovirus-based expression constructs encoding wild type, constitutively active, and dominant negative Akt, Myc-TSC1, and TSC2-Xpress have been described (3, 17). TSC2-7A and

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<sup>1</sup> The abbreviations used are: TSC, tuberous sclerosis complex; PI3K, phosphatidylinositol 3-kinase; HA, hemagglutinin; IGF, insulin-like growth factor; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; HEK, human embryonic kidney; GST, glutathione S-transferase; CDK, cyclin-dependent kinase.

TSC2-7D mutant constructs were created using a QuikChange multiple site-directed mutagenesis kit (Stratagene). Constitutively active PI3K (p110\*) was provided by Julian Downward (London, UK).

**Cell Culture, Transfection, and Flow Cytometry**—Human embryonic kidney (HEK) 293 and HeLa cells were obtained from the American Type Culture Collection. EEF4 (TSC2-positive) and EEF8 (TSC2-negative) cells were derived from Eker rat embryos homozygous for the wild type and the Eker-mutant TSC2 gene, respectively (8). All cells were grown either in Dulbecco's modified Eagle's medium or in RPMI 1640 medium, both supplemented with 10% calf serum and antibiotics. Cell transfections were performed using LipofectAMINE Plus. For cytofluorometric analyses, cells were harvested by trypsinization, fixed, and analyzed on a FACScan.

**Immunoprecipitation, Immunoblotting, and in Vitro Kinase Assay**—For immunoprecipitation, lysates were incubated with the appropriate antibody (as noted in the figure legends) in the presence of protein A-protein G (2:1)-agarose beads. The beads were washed with lysis buffer. The immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Detection of antigen-bound antibody was carried out with the ECL System. Protein kinase assays were performed as described previously (18).

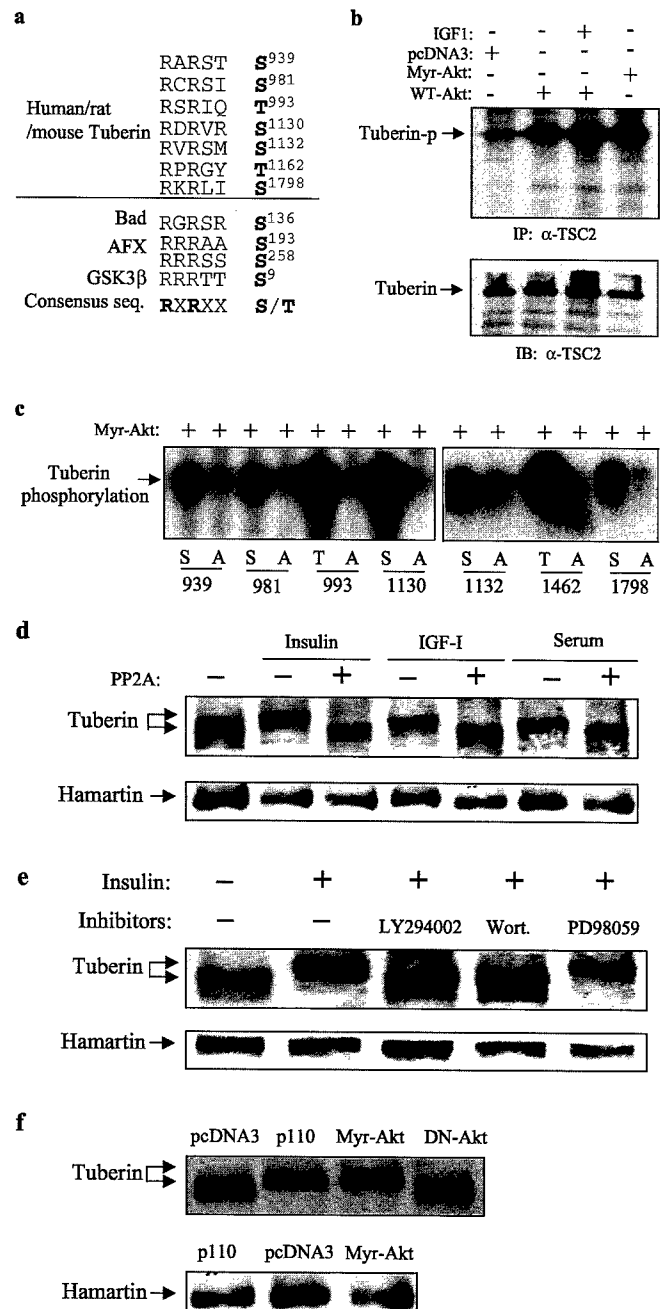
**Pulse-chase Experiments**—Prior to radioactive labeling normal culture medium was removed, and cells were washed twice with phosphate-buffered saline and refed with minimum Eagle's medium lacking methionine but supplemented with 10% dialyzed fetal bovine serum and 300  $\mu$ Ci of Tran<sup>35</sup>S-label per plate. After 60 min of labeling, cells were lysed and immunoprecipitated with anti-TSC1, -TSC2, or -p27 antibody. The immunoprecipitates were separated by SDS-PAGE gel. Gels were dried and autoradiographed. Quantification of bands was performed with a PhosphorImager.

**In Vivo [<sup>32</sup>P]orthophosphate Cell Labeling**—COS7 cells were transfected with pcDNA3-TSC2 together with or without constitutively active Akt and labeled with [<sup>32</sup>P]orthophosphate (0.5 mCi/ml) in minimum Eagle's medium without phosphate for 4 h. Tuberin was immunoprecipitated with anti-TSC2 antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membranes. Phosphorylated tuberin was detected by autoradiography.

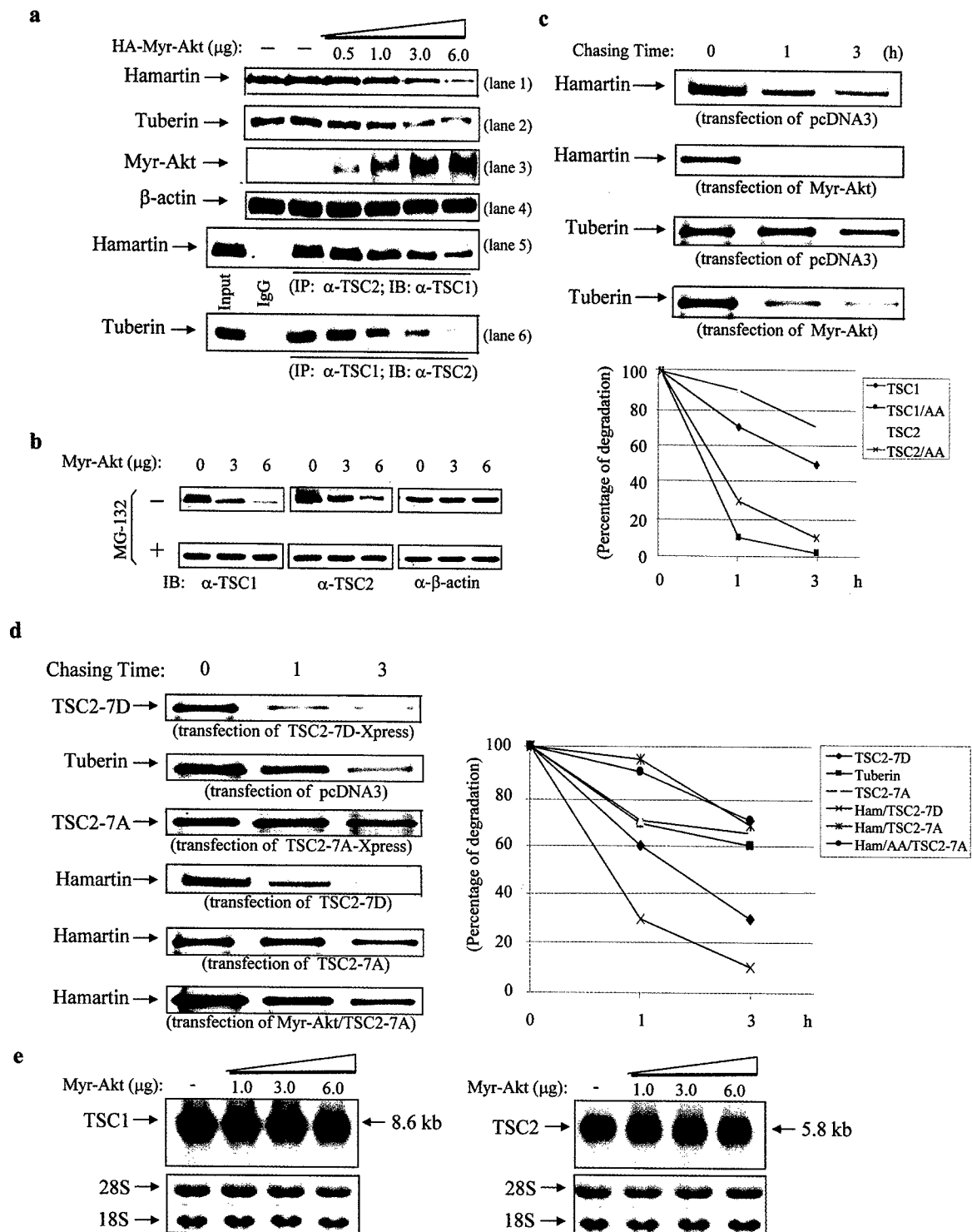
## RESULTS

**Tuberin Is a Physiological Substrate of Akt**—Recent studies demonstrated that tuberin is phosphorylated at serine and tyrosine residues in response to serum, phosphatase inhibitors, and anisomycin and that the phosphorylated tuberin regulates its interaction with hamartin (8, 9). However, the kinases that are responsible for phosphorylation of tuberin are currently unknown. Because tuberin contains seven Akt phosphorylation consensus sites that are very conserved between human, rat, and mouse as well as four that are also found in *Drosophila* (Fig. 1a), we examined the possibility of Akt phosphorylation of tuberin. *In vivo* [<sup>32</sup>P]orthophosphate cell-labeling experiments revealed that constitutively active Akt and IGF1-induced Akt significantly phosphorylate tuberin (Fig. 1b). To explore which sites on tuberin are potentially phosphorylated by Akt, *in vitro* kinase assay was carried out using wild type and mutant (converting S/T to alanine) GST fusion proteins for each of seven Akt putative phosphorylation sites as substrate. As shown in Fig. 1c, Akt can highly phosphorylate fusion proteins containing all seven serine and threonine sites of tuberin but not their mutants. We therefore conclude that tuberin is a physiological substrate of Akt.

**The PI3K/Akt Pathway, but Not the MAPK Pathway, Mediates Insulin, IGF1, and Serum-induced Tuberin Phosphorylation**—Because genetic studies of the *dTsc* complex in *Drosophila* have demonstrated that *dTsc1/dTsc2* antagonized insulin signaling in cell growth (10–13), we next examined whether insulin and IGF1 induce hamartin-tuberin phosphorylation and whether Akt mediates this action. Western blotting analyses showed that tuberin, but not hamartin, was phosphorylated upon insulin, IGF1, or serum stimulation in HeLa cells as demonstrated by gel mobility shift (Fig. 1d). The phosphorylation was abrogated by treatment with phosphatase PP2A or PI3K inhibitors, LY294002, and wortmannin, but not by MAPK



**FIG. 1. Akt phosphorylates tuberin *in vitro* and *in vivo* and mediates insulin- and IGF1-induced tuberin phosphorylation.** *a*, comparison of the putative Akt phosphorylation sites in tuberin with the sequences of phosphorylation sites of known Akt substrates. The phosphorylated residues are labeled by number, and a consensus sequence is denoted below. *b*, *in vivo* [<sup>32</sup>P]orthophosphate labeling HeLa cells transfected with constructs immunoprecipitated with anti-TSC2 antibody are indicated at the top. Immunoprecipitates were separated by SDS-PAGE, transferred to membrane, exposed to the film (upper panel), and detected by anti-TSC2 antibody (bottom panel). WT-Akt, wild type Akt. *c*, *in vitro* kinase assay analysis of constitutively active Akt (Myr-Akt) immunoprecipitates using each of the GST-fused seven Akt phosphorylation sites and their alanine (A) mutants as substrate indicated at the bottom. *d–f*, Western blot analysis of tuberin in HeLa cells that were serum-starved overnight and stimulated with or without insulin, IGF1, or serum for 15 min. Tuberin was immunoprecipitated, treated with phosphatase PP2A, and immunoblotted with anti-tuberin antibody. Electrophoretic mobility shift of tuberin, *i.e.* the phosphorylated form of tuberin, was observed upon insulin, IGF1, or serum stimulation and was abrogated by treatment with PP2A (*d*) or PI3K inhibitors (LY294002 and wortmannin) for 20 min (*e*). *f*, HeLa cells were transfected with constitutively active p110, Akt, and DN-Akt and immunoblotted with anti-TSC2 (top) and -TSC1 (bottom) antibodies.



**FIG. 3. Akt phosphorylation of tuberin induces degradation of hamartin and tuberin.** *a*, HeLa cells were transfected with HA-Myr-Akt. A portion of the cell lysate was subjected to Western blot analysis using anti-TSC1 (panel 1), -TSC2 (panel 2), -HA (panel 3), and -β-actin (panel 4) antibodies. The rest were immunoprecipitated with anti-TSC2 antibody and detected with anti-TSC1 antibody (panel 5) or vice versa (panel 6). *b*, constitutively active Akt-transfected HeLa cells were pretreated with or without MG132 for 2 h, lysed, and subjected to immunoblotting analyses with anti-TSC1, -TSC2, and -β-actin antibodies. *c*, pulse-chase analyses of degradation of hamartin and tuberin. TSC2-positive EE4 cells were transfected with plasmids indicated at the bottom of each panel, labeled with [<sup>35</sup>S]methionine, chased at indicated times, and immunoprecipitated with anti-TSC1 or -TSC2 antibodies. The immunoprecipitates were separated by SDS-PAGE, exposed, and quantified. *d*, phosphomimetic TSC2-7D promotes and nonphosphorylatable TSC2-7A inhibits the degradation of hamartin and tuberin. TSC2-deficient EE4 cells were transfected with indicated expression plasmids (bottom of each panel) and chased at indicated times after labeling with [<sup>35</sup>S]methionine. Immunoprecipitations were performed with anti-TSC1, -TSC2, or -Xpress antibodies. Graphical presentations show the normalized density of hamartin and tuberin degradation from 100%. *e*, ectopic expression of constitutively active Akt does not affect mRNA levels of TSC1 and TSC2. HeLa cells were transfected with increasing amounts of constitutively active Akt. After 48 h of transfection, total RNAs were isolated and subjected to Northern blot analyses with [<sup>32</sup>P]dCTP-labeled TSC1 (left) or TSC2 (right) cDNA probe. Bottom panels indicate equal loadings.

its degradation (20, 21). To examine the effects of Akt phosphorylation of tuberlin on p27<sup>kip1</sup> expression, pulse-chase analyses were performed with TSC2-deficient EE8 cells that were transfected with TSC2, TSC2/Mry-Akt, TSC2-7D, or TSC2-7A. As shown in Fig. 4a, expression of constitutively active Akt abrogated the ability of stabilization of p27<sup>kip1</sup> by tuberlin. P27<sup>kip1</sup> degraded rapidly in phosphomimic TSC2-7D-transfected cells as compared with the cells expressing wild type TSC2/constitutively active Akt. In contrast, the cells expressing TSC2-7A exhibited similar degradation rate of p27<sup>kip1</sup> to wild type TSC2-transfected cells. Moreover, expression of TSC2-7A abrogated constitutively active Akt-induced p27<sup>kip1</sup> degradation (Fig. 4a). These data indicate that degradation of p27<sup>kip1</sup> is regulated by Akt phosphorylation of tuberlin.

Because G<sub>1</sub>/S CDK 2 is a major target of p27<sup>kip1</sup> (21), we next examined whether Akt overrides tuberlin-inhibited CDK2 activity. Consistent with previous reports (21, 22), expression of wild type TSC2 inhibited CDK2 activity in a dose-dependent manner. However, constitutively active Akt abrogated TSC2-inhibited CDK2 activity (Fig. 4b). Phosphomimic TSC2-7D lost the ability to inhibit CDK2 activity, whereas expression of nonphosphorylatable TSC2-7A displayed the same effects as wild type TSC2 (Fig. 4b).

Because CDK2 is a major regulator of cell growth and G<sub>1</sub>/S transition of the cell cycle, we further examined the effects of Akt phosphorylation of tuberlin on cell proliferation measured by cell growth and thymidine incorporation. As shown in Fig. 4, c and d, expression of TSC2 or TSC2-7A in TSC2-deficient EE8 cells inhibited cell growth and DNA synthesis as compared with the cells transfected with vector alone. However, cells expressing constitutively active Akt or phosphomimic TSC2-7D significantly enhanced cell growth and thymidine incorporation. Consistent with previous findings, the number of cells at the G<sub>0</sub>-G<sub>1</sub> phase of the cell cycle was increased in the cells expressing TSC2. Expression of TSC2-7A displayed a similar effect on cell cycle. In contrast, constitutively active Akt overrode wild type TSC2 action. The cell number of the S phase was increased in constitutively active Akt/TSC2- or phosphomimic TSC2-7D-transfected cells (Fig. 4e). These data indicate that Akt phosphorylated tuberlin lost its tumor suppressor function at least in part by inducing p27<sup>kip1</sup> degradation.

#### DISCUSSION

Recent studies have demonstrated that phosphorylation of hamartin and/or tuberlin may play an important role in the formation of the tuberlin-hamartin complex. Tuberlin is phosphorylated at serine and tyrosine residues, and a disease-related TSC2 tyrosine 1571 mutation (Y1571H) nearly abolishes tuberlin tyrosine phosphorylation and disrupts tuberlin-hamartin binding, implying that the phosphorylation of tyrosine 1571 of TSC2 is required for tuberlin-hamartin complex formation (8, 9). Our study, however, shows that phosphorylation of tuberlin by Akt and mitogenic factors (insulin and IGF1) abrogates hamartin-tuberlin tumor suppressor activity without interfering with binding but by inducing degradation of both proteins through the proteasome pathway. Therefore, we provide a new paradigm for regulation of the TSC1/TSC2 tumor suppressor pathway.

In addition to the Forkhead transcription factor family (16, 22), tuberlin is the second Akt downstream target that has been uncovered by genetic studies so far. In this study, we present molecular evidence that tuberlin is a direct physiological substrate of Akt by demonstrating that Akt binds to and phosphorylates tuberlin. It has been documented that Akt induces cell cycle progression and cell proliferation through transcription repression and degradation of p27<sup>kip1</sup> (23, 24). Akt inhibition of p27<sup>kip1</sup> transcription is achieved by Akt phosphorylation of a

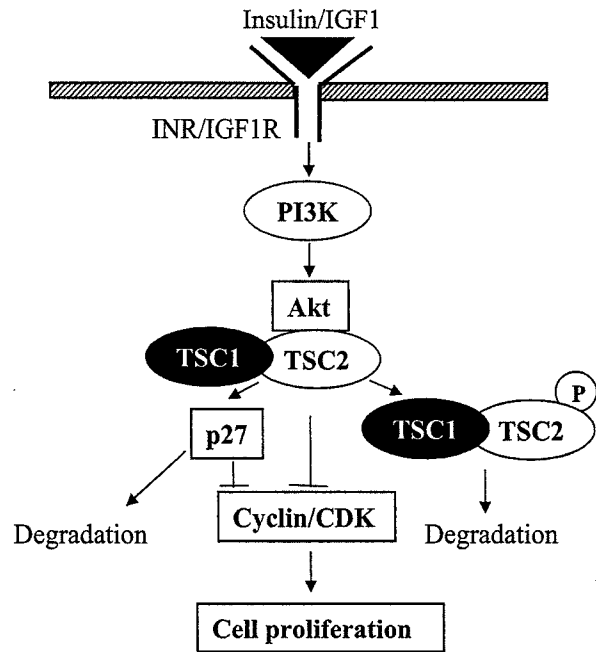


FIG. 5. Schematic illustration of negative regulation of the TSC tumor suppressor complex by PI3K/Akt.

Forkhead transcription factor, AFX, leading to the decrease of p27<sup>kip1</sup> promoter activity (24). However, the mechanism of Akt degradation of p27<sup>kip1</sup> is unclear. Tuberlin was revealed to stabilize p27<sup>kip1</sup> by maintaining p27<sup>kip1</sup> in the nucleus (20). We observed in this study that Akt attenuates the tuberlin action but does not induce translocation of p27<sup>kip1</sup> from nuclear to cytoplasm (data not shown). Previous studies have shown that three isoforms of Akt share almost the same upstream regulators and downstream targets. Similarly, we have observed that Akt1, Akt2, and Akt3 all phosphorylate and interact with tuberlin, even though Akt2 displays a slightly higher binding affinity to tuberlin. The model in Fig. 5 illustrates the mechanism through which the PI3K/Akt pathway mediates insulin and IGF1 signals to down-regulate hamartin-tuberlin function by phosphorylation of tuberlin. Our results define a possible new mechanism through which Akt induces cell proliferation and transformation by inhibiting TSC1/TSC2 tumor suppressor functions.

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**BIOGRAPHICAL SKETCH**

Provide the following information for the key personnel in the order listed on page 1 of the Detailed Cost Estimate form for the initial budget period.

NAME		POSITION TITLE	
Jin Q. Cheng		Associate Professor	
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Capital Medical University, Beijing, P.R.C.	M.D.	1986	Medicine
Paris University XIII, Paris, France	Ph.D	1994	Molecular Cell Biology
Fox Chase Cancer Center, Philadelphia	Postdoc.	1996	Molecular Tumor Genetics

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED THREE PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.**

**Professional Experience:**

1994-1996 **Postdoctoral Associate**, Fox Chase Cancer Center, Philadelphia, PA  
 1996-1997 **Staff Scientist**, Fox Chase Cancer Center, Philadelphia, PA  
 1997-2001 **Assistant Professor**, H. Lee Moffitt Cancer Center and Research Institute, Department of Pathology, University of South Florida College of Medicine, Tampa, FL  
 2001-present **Associate Professor**, H. Lee Moffitt Cancer Center and Research Institute, Department of Pathology, University of South Florida College of Medicine, Tampa, FL

**Honors and Awards:**

1994-1997 Special Fellow Award from The Leukemia Society of America  
 1994 Young Investigator Award of the International Association for the Study of Lung Cancer (IASLC),  
 1988-1990 Fellowship for pre-doctoral training, French Cancer Research Association (L'ARC France)

**Publications (Partial Listing):**

Dan HC, Coppola D, Jinag K, Liu A, Hamilton AD, Nicosia SV, Sebti SM, **Cheng JQ**. Phosphatidylinositol-3-OH Kinase/Akt and Survivin Pathways as Critical Targets for Geranylgeranyltransferase I Inhibitors Induced Apoptosis. *Oncogene*, *In press*  
 Leung BM, Fraser M, Yan X, Dan HC, **Cheng JQ**, Tsang BK. Akt and p53 Are Determinants of Xiap-Mediated Chemoresistance in Human Ovarian Cancer. *Cancer Res.*, *In press*  
 Sun M, Yang L, Feldman RI, Sun X, Jove R, Nicosia SV, **Cheng JQ**. Activation of Phosphatidylinositol-3-OH Kinase/Akt Pathway by Androgen through interaction of p85 $\alpha$ , AR and Src. *J. Biol. Chem.* *In press*  
 Chen T, Yang I, Irby R, Shain KH, Wang HG, Quackenbush J, Coppola D, **Cheng JQ**, Yeatman TJ. Regulation of caspase expression and apoptosis by adenomatous polyposis coli. *Cancer Res.* 63:4368-4374, 2003.  
 Yuan Z, Feldman RI, Sussman GE, Coppola D, Nicosia SV, **Cheng JQ**. AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: Implication of AKT2 in chemoresistance. *J. Biol. Chem.* 278:23432-13440, 2003.  
 Gritsko TM, Coppola D, Paciga JE, Yang L, Sun M, Shelley SA, Fiorica JV, Nicosia SV, **Cheng JQ**. Activation and Overexpression of Centrosome Kinase BTAK/Aurora-A in Human Ovarian Cancer. *Clin Cancer Res.* 9:1420-1426, 2003.



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- Cheng JQ**, Jiang X, Fraser M, Li M, Dan HC, Sun M, Tsang BK. Role of X-linked Inhibitor of Apoptosis Protein in chemoresistance in Ovarian Cancer: Possible involvement of the PI 3-Kinase/Akt Pathway. *Drug Resist Updat.* 5:131-146, 2002.
- Sun M, Paciga, JE, Feldman RI, Yuan Z, Shelley SA, Nicosia SV, **Cheng J.Q**. Phosphatidylinositol-3-OH kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor  $\alpha$  (ER $\alpha$ ) via interaction between ER $\alpha$  and PI3K. *Cancer Res.*, 61:5985-5991, 2001.
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## Research Support

### Active

R01 CA 077935-06 (P.I. Cheng, J.Q.)

NCI	\$175,000	20%
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The Role of AKT2 Oncogene in Human Oncogenesis

The major goals of this project are... to examine (1) transcriptional regulation of AKT2 and characterization of AKT2 promoter, (2) the effects of AKT2-interaction protein, NAB, on AKT2 signaling, (3) the effects of AKT2 phosphorylation of TSC2 on cell cycle control, and (4) AKT2 inducible genes identified by cDNA array.

R01 CA 89242 (P.I. Cheng, J.Q.)

NCI	6/1/01 – 5/31/06	
	\$157,500	20%

AKT1 Oncogene in Carcinogenesis

The major goals of this project are... to examine the importance of AKT1 in prostate carcinogenesis and the effects of AKT1-interaction proteins, AP $\alpha$ B and PMSB4, on AKT1 survival and growth.

DAMD17-00-0559 (P.I. Cheng, J.Q.)

DOD	9/1/00 – 8/31/03	
	\$100,000	15%

**Phosphoinositide 3-kinase/AKT1 pathway and human ovarian cancer**

The major goals of this project are...to examine the incidence/clinical significance of *PI3K/AKT1* alterations in ovarian cancer and the role of overexpression of active and wild type PI3K and AKT1 in ovarian surface epithelial (OSE) cell transformation *in vitro* and *in vivo*.

DAMD17-01-1-0394 (P.I. Cheng, J.Q.) 9/1/01 – 8/31/04

DOD \$100,000 15%

**AKT2 Oncogene and Phosphoinositide 3-kinase in Human Breast Cancer**

The major goals of this project are...to examine the incidence and clinicopathological significance of PI3K/AKT2 alterations in breast cancer and (2) the role of PI3K/AKT2 in chemoresistance and tomaxifen resistance.

DAMD17-02-1-0671 (project leader, Cheng J.Q..) 9/1/02 – 8/31/06

DOD \$83,000 10%

**Cell Growth and Survival in Ovarian Epithelial Cancer**

The major goals of this project are...to examine the role of AKT-interaction protein, APBP, in ovarian cancer development and the mechanism of APBP in chemoresistance.

Pharmaceutical company support (P.I. Cheng, J.Q.) 6/1/99 – 5/31/04 5%

Berlex Bioscience \$85,000

**Akt as target for cancer intervention**

The major goals of this project are...to develop Akt inhibitor(s).

R01CA85709 (PI: Said M. Sebti; Co-PI: Cheng, J.Q.) 3/1/01 – 3/1/06

NIH \$125,000 10%

**AKT Survival Pathways and FTI-induced Apoptosis**

The major goals of this project are...to examine the mechanism of FTI inhibition of PI3K/AKT pathway.